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14. ABSTRACT: The objective of the project for the reporting period was to identify high affinity SLUG-regulated gene promoters from human breast cells. We over expressed 3xFLAG-tagged (C-terminal) human SLUG in the SLUG-negative MDA-MB-468 and MCF-7 cells through a lentiviral construct. By chromatin immunoprecipitation assay and Q-PCR, we identified/reconfirmed a handful of the gene promoters that indeed bind in vivo with SLUG in the human breast cells tested. They include cytokeratins 8, 18, and 19, E-cadherin, occludin, Na/K ATPase, vitamin D receptor, integrin alpha 3, PUMA, BRCA2, claudins 5, 3, 7, 11, 1, 14, and 16, desmoglein 1, and 2, PCNA, PGDH and plakoglobin gene promoters. We are employing the chromatin immuno-precipitation-DNA selection and ligation (ChIP-DSL, formerly known as ChIP-GLAS, Aviva System Biology) technique for further analysis. We evaluated the mRNA levels of these potentially SLUG-regulated genes in invasive and non-invasive human breast tumor cells. We have designed DNA decoys against the DNA binding domain of SLUG from the claudin 7 gene promoter (highest affinity for SLUG among the promoters tested). We are currently testing whether these decoys can block the binding of SLUG in vivo to its target promoters and stimulate the expressions of SLUG-regulated genes in human breast cells.

15. SUBJECT TERMS
Breast cancer, SLUG, transcriptional silencing, molecular decoys, metastasis

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INTRODUCTION

SLUG is a zinc finger transcriptional repressor protein that mediates its action through the binding to E2-box sequences (5′-CACCTG-3′) at the promoters of its target genes. SLUG and the other member of its family, SNAIL, are known to down regulate the expressions of many cell adhesion molecules (1-5). While SNAIL is reported to play a major role in these regulations in the non-breast cells (1-3), SLUG seems to be the major player in the human breast cells (4, 5). We have reported that SLUG also regulates the expression of the tumor suppressor protein BRCA2 and cytokeratins 8 and 19 in human breast cells (4, 5). An interesting aspect to add is that all the highly invasive human breast tumor cell lines express high levels of SLUG whereas the non-invasive breast cells are either SLUG negative or express very little of this protein. We postulated that a high level of SLUG protein in the breast epithelial cells inhibits the expression of BRCA2 thus promoting unfettered growth of the cells as well as the inhibition of the cell adhesion molecules by SLUG helps the proliferating tumor cells to transform to the mesenchymal cells and ultimately to metastasize. We have planned a series of experiments to **(i)** identify SLUG-target gene promoters to design high affinity SLUG-binding ds-DNA decoys; **(ii)** characterize the co-repressor binding domains of SLUG to design high affinity peptide aptamers that will block the binding of the co-repressors to the SLUG protein; and **(iii)** deliver the siRNA, ds-DNA decoy and peptide aptamers to human breast tumor cells to efficiently knock down the SLUG activity and to evaluate the effects of this ablation on the proliferation, invasiveness and metastasis of these cancer cells in 3D-tissue culture and mice models.

BODY

Task outlined in the approved Statement of Work for this period of the project

Task#1. Identification and analysis of the promoters of SLUG-regulated genes to design high affinity SLUG-binding ds-DNA decoys (Months 1-15):

Our progress/accomplishments associated with the task are as follows:

1. We developed 3xFLAG-tagged SLUG expressing SLUG negative human breast cells for better ChIP analysis. We over expressed 3xFLAG-tagged (C-terminal) human SLUG in the SLUG-negative MDA-MB-468 and MCF-7 cells through a doxycycline inducible CMV promoter in a lentiviral construct (Invitrogen). Data from the MCF7 cells are shown in Fig. 1. Both the SLUG mRNA and SLUG protein levels are increased in the presence of doxycycline (1 μg/ml). The invasiveness of the cells, as was measured using Boyden chambers, was increased in the presence of doxycycline but decreased when siRNA against SLUG (4) was used in the presence of dox (Fig. 2).

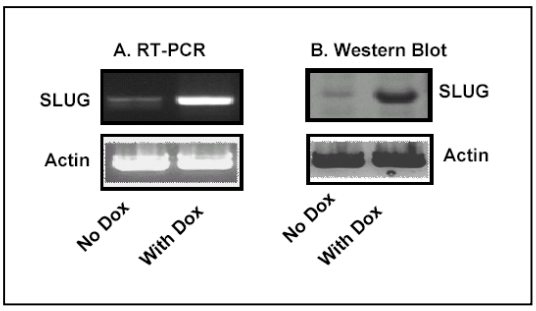

Fig. 1. Evaluation of doxycycline-induced over expression of 3XFLAG-tagged SLUG in MCF7 cells. (A) RT-PCR data. (B) Western blotting with anti-FLAG and anti-actin antibodies. Performed as described in ref. 4

We got similar data with MDA-MB-468 cells (data not shown). This 3xFLAg tagged SLUG is an improvement for the subsequent ChIP analysis because the SLUG antitibody that we developed or that are available commercially (Santa Cruz biotech) are not as efficient as FLAG antibody raised against 3xFLAG epitope (Sigma). Development of these SLUG expressing cell lines was critical for the subsequent identification SLUG-target genes in the human breast cells.

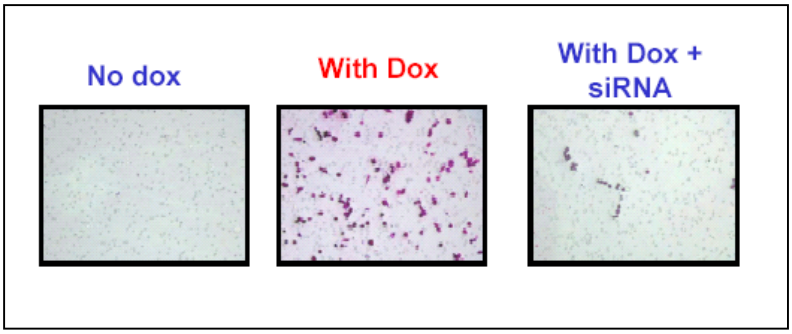

Fig. 2. Assay for *in vitro* invasiveness of the SLUG over expressing MCF7 cells. Assay was done in Boyden chambers (6).

2. We have identified/reconfirmed several gene promoters as those binding to SLUG *in vivo*. By chromatin immunoprecipitation assay and Q-PCR, we identified/reconfirmed a handful of the gene promoters that indeed bind *in vivo* with SLUG in the human breast cells tested. They include cytokeratins 8, 18, and 19, E-cadherin, occludin, Na/K ATPase, vitamin D receptor, integrin alpha 3, PUMA, BRCA2, claudins 1, 3, 5, 7, 11, 14, and 16, desmoglein 1, and 2, PCNA, PGDH and plakoglobin gene promoters. We are employing the chromatin immuno-precipitation-DNA selection and ligation (ChIP-DSL, formerly known as ChIP-GLAS, Aviva System Biology) technique for further analysis (7, 8).

3. Expressions of the SLUG target genes are down regulated in the invasive human breast cancer cells. We evaluated the mRNA levels of these potentially SLUG-regulated genes in invasive and non-invasive human breast tumor cells. We used MCF7 and MDA-MB-468 cells as non-invasive cells and MDA-MB-231 and BT549 cells as invasive cells (4, 5). Table 1 shows the data for the relative expressions of the 22 genes as was determined by real-time RT-PCR analyses.

4. Claudin 7 gene promoter has many E2 boxes that bind to SLUG and inhibit claudin 7 gene expression. We studied the promoter of human claudin 7 (CLDN7) gene is greater details to develop molecular decoy because (i) this promoter binds with high affinity to SLUG as was determined by Q-PCR of the ChIP DNA obtained with antiFLAG antibody from the SLUG-over expressing MCF7 cells as compared to total CLDN7 promoter DNA present; and (ii) this promoter has at least 7 E2-box sequences (Fig. 3) that potentially can bind SLUG.

CAGGCGCACCTGTTGGGAAGAAAGGAAGGGGCTTCCCGGTGTTCGAGGGAAATCCAGTCCGGAGGGGCTGACTCGGA
GCTTGGGACTCCTGGGGAGCCACCGCCTCCTCCCCAGCGGCGGTCAAAACCGGGCAAGCGAAGGGGCGTGACCCTGG
TGCTCAGGTTTCTTCCTCCTCACCTGGGCAAGGAGGGGTGGGGGCCACGACTTCCGGTTCAGGTGAGTGTCCCTTCG
GTGACGTCAGGTCATCCTCGGCCGCCCCTCCGGTCCCGCCTCCCCCTCCCGCGCTCCCGGGGCGCGCGGGCCGCGCC
CCCGACGCCCTACATATACTCAGGTGCGCCCCACCTGTCCGCCCGCACCTGCTGGCTCACCTCCGAGCCACCTCTGC
TGCGCACCGCAGCCTCGGACCTACAGCCCAGGATACTTTGGGACTTGCCGGCGCTCAGAAACGCGCCCAGACGGCCC
CTCCACCTTTTGTTTGCCTAGGGTCGCCGAGAGCGCCCGGAGGGAACCGCCTGGCCTTCGGGGACCACCAATTTTGT
CTGGAACCACCCTCCCGGCGTATCCTACTCCCTGTGCCGCGAGGCCATCGCTTCACTGGAGGGGTCGATTTGTGTGT
AGTTTGGTGACAAGATTTGCATTCACCTGGCCCAAACC

Fig. 3. Nucleotide sequences of the human CLDN7 gene promoter used in this study. The E2 boxes are highlighted in red and cyan. The red boxes are complementary to the cyan boxes. The yellow shading indicates the reported (Genbank) transcriptional start site.

Table 1. Fold decrease (as compared to that in the normal human breast cell MCF10A) in the mRNAs of the SLUG-target genes in the non-invasive and the invasive breast cancer cells (Results are mean ± SE, n=6).

Gene	Non-Invasive cells		Invasive cells	
	MCF7	MDA-MB-468	MDA-MB-231	BT549
Cytokeratin 8	1.2 ± 0.3	1.0 ± 0.1	6.1 ± 0.4	7.1 ± 0.1
Cytokeratin 18	1.1 ± 0.1	1.2 ± 0.4	3.0 ± 0.3	3.2 ± 0.1
Cytokeratin 19	1.4 ± 0.1	1.3 ± 0.3	4.7 ± 0.2	4.9 ± 0.2
E-cadherin	1.4 ± 0.2	1.2 ± 0.2	3.8 ± 0.3	4.2 ± 0.4
Occludin	1.3 ± 0.1	1.1 ± 0.2	6.3 ± 0.1	5.9 ± 0.4
Na/K ATPase	1.2 ± 0.3	1.2 ± 0.3	3.8 ± 0.1	3.6 ± 0.3
VDR	1.3 ± 0.3	1.1 ± 0.1	5.1 ± 0.3	5.8 ± 0.1
Integin a3	1.0 ± 0.2	1.3 ± 0.2	3.2 ± 0.1	3.6 ± 0.2
PUMA	1.0 ± 0.1	1.0 ± 0.2	5.5 ± 0.3	5.2 ± 0.2
BRCA2	1.2 ± 0.1	1.1 ± 0.3	4.6 ± 0.4	4.5 ± 0.2
Claudin 1	1.3 ± 0.3	1.1 ± 0.4	4.2 ± 0.5	4.4 ± 0.5
Claudin 3	1.1 ± 0.1	1.0 ± 0.09	6.7 ± 0.4	6.2 ± 0.1
Claudin 5	1.1 ± 0.2	1.1 ± 0.1	3.2 ± 0.3	3.7 ± 0.3
Claudin 7	1.0 ± 0.1	1.2 ± 0.3	8.1 ± 0.1	9.3 ± 0.4
Claudin 11	1.1 ± 0.1	1.0 ± 0.1	5.2 ± 0.3	5.7 ± 0.3
Claudin 14	1.1 ± 0.3	1.1 ± 0.1	4.3 ± 0.4	4.9 ± 0.2
Claudin 16	1.1 ± 0.2	1.0 ± 0.2	4.5 ± 0.3	4.1 ± 0.3
Desmoglein 1	1.3 ± 0.1	1.0 ± 0.3	5.5 ± 0.4	4.2 ± 0.1
Desmoglein 2	1.1 ± 0.1	1.0 ± 0.3	3.6 ± 0.1	4.9 ± 0.2
PCNA	1.1 ± 0.03	1.2 ± 0.2	6.2 ± 0.1	5.1 ± 0.3
PGDH	1.3 ± 0.2	1.2 ± 0.1	4.4 ± 0.7	6.2 ± 0.9
Packoglobin	1.0 ± 0.1	1.1 ± 0.08	3.1 ± 0.3	3.3 ± 0.2

We found that CLDN7 is indeed down regulated in the SLUG-positive invasive human breast cancer cells (Figs. 4-6). We created individual E2-box mutants of this promoter by site-directed mutagenesis (4) and evaluated the activity of this promoter in the SLUG-expressing MCF7 cells. In the presence of doxycycline inducer the activity of the repressed when there is no mutation or mutation is in the E2 boxes 1, 2, 3 and 7. But the promoter sequences mutated at the E2 boxes 4. 5 and 6 are not repressed (Fig. 7).

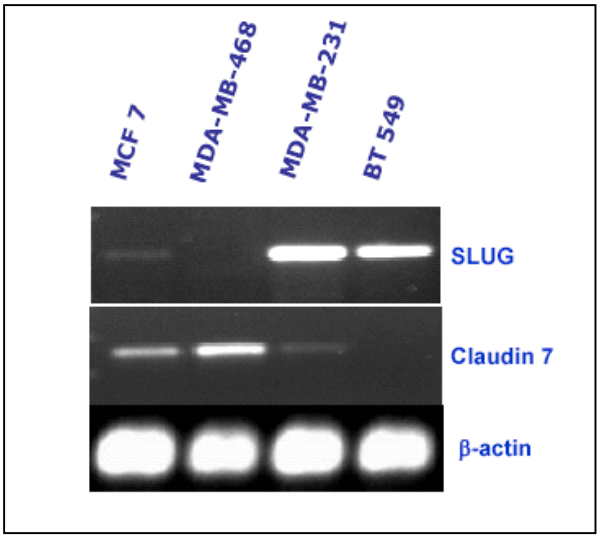

Fig. 4. Evaluation of Claudin 7 gene expression in the non-invasive (MCF7 and MDA-MB-468) and invasive (MDA-MB-231 and BT549) cells. RT-PCR data is shown. Invasiveness of the cells are based on Boyden chamber assays. Beta active mRNA was used as a control.

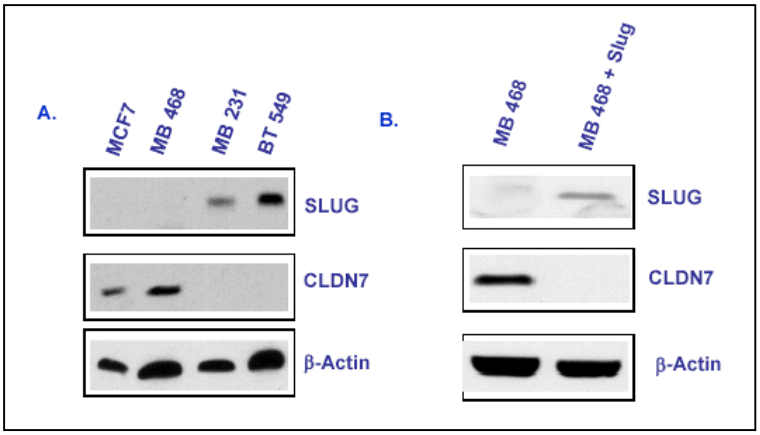

Fig. 5. Fig. 4. Evaluation of Claudin 7 protein expression in the non-invasive (MCF7 and MDA-MB-468) and invasive (MDA-MB-231 and BT549) cells. Western blotting data are shown. (A) Data with wild-type cells. (B) Data with SLUG-over expressing MDA-MB-468 cells. Beta actin was used as a loading control.

5. Molecular decoy designed from the human CLDN7 gene promoter prevents CLDN7 promoter activity in the transfected human breast cells. Based on the data described above, we designed (see Fig. 8) a 41 bp molecular decoy (-28 - +13 encompassing E2 boxes 4, 5 and 6) to test whether that can block the function of SLUG in human breast cells. We initially annealed two 38 nt long complementary oligos to create double stranded DNA with 3 nt 5'-overhangs at each end. We then filled in those ends using α-S-dNTPs to create terminally phosphorothioated 41 bp DNA molecules. This treatment made the DNA resistant to 5'-exonucleases. Similarly, we created 41 bp control molecules in which the E2-boxes are mutated hence non-functional (Fig. 8). The experimental decoy, but not the control decoy, could inhibit the function of SLUG in SLUG over expressing MCF7 cells when evaluated by the expression of Renilla luciferase (Rluc) activities in the cells transfected with a reporter plasmid containing Rluc gene behind human CLDN7 gene promoter (Fig. 9). We are currently testing whether this decoy can specifically inhibit the expressions of SLUG target genes in the invasive breast cancer cells.

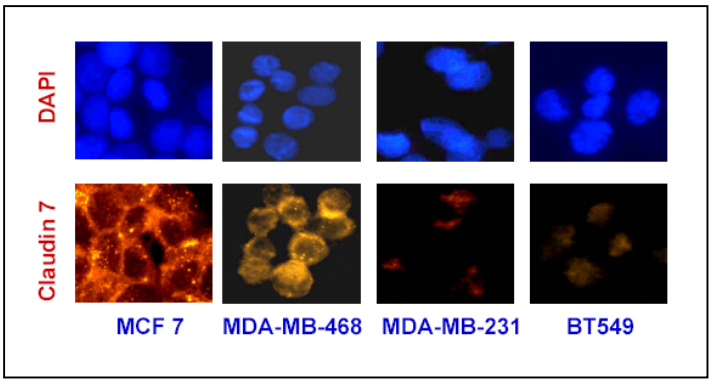

Fig. 6. Immunofluorescence microscopy for the expression of Claudin 7 in the invasive and non-invasive human breast cells.

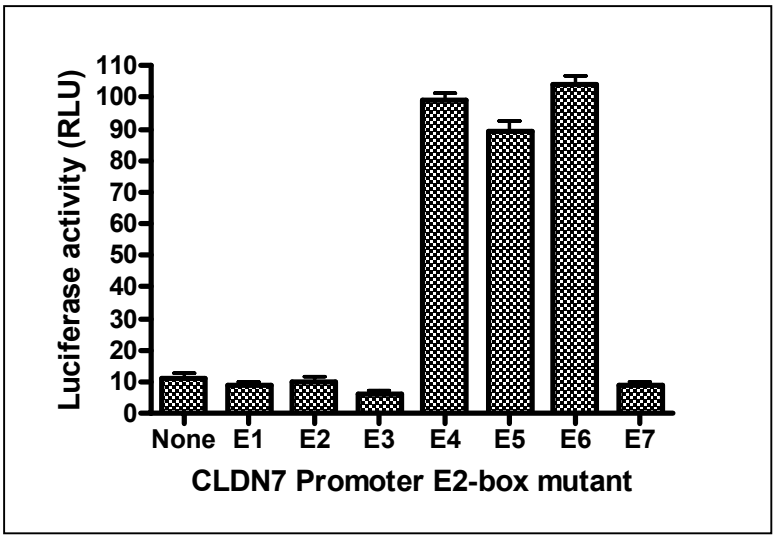

Fig. 7. Evaluation of the effect of mutation (CACCTG is changed to AACCTA) of different E2-box elements of the human CLDN7 gene promoter on its activity in the SLUG over expressing MCF7 cells.

A. Experimental decoy

5′-ATACTCAGGTGCGCCCCACCTGTCCGCCCGCACCTGCTGGC-3′
3′-TATGAGTCCACGCGGGGTGGACAGGCGGGCGTGGACGACCG-5′

B. Control mutated decoy

5′-ATACTTAGGTTCGCCCAACCTATCCGCCCGAACCTACTGGC-3′
3′-TATGAATCCAAGCGGGTTGGATAGGCGGGCTTGGATGACCG-5′

Fig. 8. (A) Nucleotide sequences of the experimental molecular decoy that was designed from the human CLDN7 promoter sequences. The highlighted (red and cyan) are the E2 boxes 4, 5 and 6 sequences. The yellow shading indicates the transcribed regions of the CLDN7 gene. **(B)** Nucleotide sequences the control molecular decoy in which the E2-boxes are mutated.

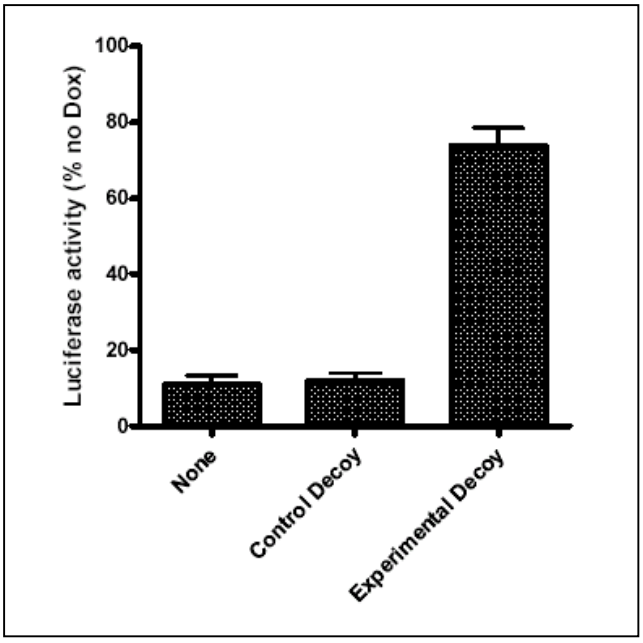

Fig. 9. Effect of the molecular decoy (25 μM) on the CLDN7 gene promoter activity in doxycycline induced SLUG-over expressing MCF7 cells. Data are mean + SE, n=6.

KEY RESEARCH ACCOMPLISHMENTS

- We have identified 22 genes in the human breast cells that are directly regulated by the transcriptional silencer protein SLUG.
- All these 22 genes are down regulated in invasive, SLUG-positive breast cancer cells in comparison to the SLUG-negative non-invasive breast cancer cells.
- By Q-PCR of the ChIP DNA we identified human claudin 7 (CLDN7) gene promoter is the most avid promoter for SLUG among the 22 gene promoters we studied.
- We identified three E2 box sequences in the CLDN7 gene promoter to be essential for the SLUG-mediated repression of CLDN7 gene.
- A 41-bp terminally phosphorothioated molecular decoy could inhibit SLUG function in the human breast cells.

REPORTABLE OUTCOMES: The research performed directly or indirectly contributed to the following publications and poster abstracts.

Publication:

1. Bailey, C. K., Misra, S., Mittal, M. K., and **Chaudhuri, G**. (2007) Human SLUG does not directly bind to CtBP1. ***Biochem. Biophys. Res. Commun***. **353**, 661-664.

Meeting abstracts:

1. Mittal, M. K., Bailey, C. K., Misra, S. and **Chaudhuri, G.** (2006) SLUG-dependent modulation of Claudin 7 gene expression in metastatic human breast tumor cells. **Presented as a**

poster at the ASBMB Transcription Meeting at Kiawah Island Resort in SC on Nov 2-6, 2006.

2. Mittal, M. K., Bailey, C. K., Myers, J., Misra, S. and **Chaudhuri, G.** (2007) Differential contribution of distinct E2-box elements in the dual regulation of human claudin 7 gene promoter by SNAIL and SLUG in human breast cells. **Presented as a poster in the Annual Meeting of American Association for Cancer Research in Los Angeles, CA, April 14-18, 2007.**

3. Bailey, C. K., Misra, S., Mittal, M. K., and **Chaudhuri, G.** (2007) Human SLUG does not directly bind to CtBP1. **Presented as a poster in the Annual Meeting of American Association for Cancer Research in Los Angeles, CA, April 14-18, 2007.**

CONCLUSION: We have identified several genes in the human breast cells that are regulated by direct binding of SLUG to their promoters. These target genes are down regulated in the invasive breast cancer cells as compared to the non-invasive cells or non-cancerous cells. This observation may suggest that SLUG-regulated inhibition of the expressions of those genes may be directly or indirectly involved in the proliferation and metastasis of the cancer cells. Double stranded DNA segments designed from the SLUG binding site of the highly sensitive human claudin 7 gene promoter will be useful to inhibit SLUG binding to its target gene promoter and thus to prevent SLUG to induce metastatic transformations of the human breast tumor cells.

REFERENCES:

1. Barrallo-Gimeno, A., and Nieto, M. A. (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development*. **132**, 3151-3161.

2. Nieto MA. (2002) The snail superfamily of zinc-finger transcription factors. *Nat*. *Rev. Mol. Cell. Biol.* **3**,155-166.

3. Hemavathy, K., Guru, S. C., Harris, J., Chen, J. D., and Ip, Y. T. (2000) Human Slug is a repressor that localizes to sites of active transcription. *Mol. Cell. Biol*. **20**, 5087-5095.

4. Tripathi, M. K., Misra, S., Khedkar, S. V., Hamilton, N., Irvin-Wilson, C., Sharan, C., Sealy, L., and Chaudhuri, G. (2005) Regulation of BRCA2 gene expression by the SLUG repressor protein in human breast cells. *J. Biol. Chem.* **280**, 17163-17171.

5. Tripathi, M. K., Misra, S., and Chaudhuri, G. (2005) Negative regulation of the expressions of cytokeratins 8 and 19 by SLUG repressor protein in human breast cells. *Biochem. Biophys. Res. Commun*. **329**, 508-515.

6. Bratt, A., Birot, O., Sinha, I., Veitonmaki, N., Aase, K., Ernkvist, M., and Holmgren, L. (2005) Angiomotin regulates endothelial cell-cell junctions and cell motility. *J. Biol. Chem*. **280**, 34859-34869.

7. Kwon, Y. S., Garcia-Bassets, I., Hutt, K. R., Cheng, C. S., Jin, M., Liu, D., Benner, C., Wang, D., Ye, Z., Bibikova, M., Fan, J. B., Duan, L., Glass, C. K., Rosenfeld, M. G., and Fu, X.

D. (2007) Sensitive ChIP-DSL technology reveals an extensive estrogen receptor alpha-binding program on human gene promoters. *Proc. Natl. Acad. Sci. U S A*. **104**, 4852-4857.

8. Garcia-Bassets, I., Kwon, Y. S., Telese, F., Prefontaine, G. G., Hutt, K. R., Cheng, C. S., Ju, B. G., Ohgi, K. A., Wang, J., Escoubet-Lozach, L., Rose, D. W., Glass, C. K., Fu, X. D., and Rosenfeld, M. G. (2007) Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell*. **128**, 505-518.

APPENDICES: One reprint and three abstracts added.

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Human SLUG does not directly bind to CtBP1

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Abstract

SLUG is a transcriptional repressor protein implicated to have major role in the oncogenesis and metastasis of human breast cells. We previously have shown by chromatin immunoprecipitation assay that human SLUG (hSLUG) is co-localized with the co-repressor protein CtBP1 as bound to the BRCA2 gene silencer [M.K. Tripathi, S. Misra, S.V. Khedkar, N. Hamilton, C. Irvin-Wilson,, C. Sharan, L. Sealy, G. Chaudhuri, J. Biol. Chem. 280 (2005) 17163–17171]. hSLUG was predicted to be binding directly to CtBP1 because of an apparent presence of CtBP1 binding site in its amino acid sequences. Here, we provide evidence through yeast two-hybrid and *in vitro* co-immunoprecipitation analyses that hSLUG does not directly interacts with hCtBP1. This observation will help in the study of the mode of action of hSLUG in human cells.

Keywords: SLUG; CtBP1; Yeast 2-hybrid; Co-immunoprecipitation; Repressor; SNAG domain

SLUG is a member of a superfamily of zinc-finger transcriptional repressors [1–3]. SLUG and its family members are involved in tumor progression [1]. SLUG is implicated to induce epithelial to mesenchymal transition in many cells [2]. The different family members are now grouped into two families: *Snail* and *Scratch*. Vertebrates have three *Snail* members: SNAIL (new name SNAIL1), SLUG (now named SNAIL2), and SNAIL3 (formerly SMUC) [2,3]. Human cells have two SCRATCH proteins: SCRATCH1 and SCRATCH2. Human breast cells we have studied so far do not express SCRATCH transcripts (M.K. Tripathi and G. Chaudhuri, unpublished). Human SLUG gene is located at chromosome 8q11, has 3 exons and 2 introns, is transcribed into a ~2.1-kb mRNA (Accession No. NM_003068), and encodes a C2H2-type zinc finger transcription factor protein with 268 amino acids [4]. The encoded protein acts as a transcriptional repressor that binds to E2-box motif (5′-CACCTG-3′) [4,5].

Although many genes have the E2-box sequences at their promoters, only the expressions of few proteins are experimentally shown to be regulated by SLUG. In human mammary epithelial cells, SLUG is shown to negatively regulate the E-cadherin [5], aromatase [6], PUMA [7], BRCA2 [8], claudin-1 [9], integrin alpha3, beta1, and beta4 [10] and, cytokeratin 8 and 19 [11] gene expressions. Other genes that are potentially down regulated inside the human breast cells by SLUG may include those of VE-cadherin, other claudins, occludins, desmoplakin, and mucin-1 [2,3]. Majority of the genes that are down regulated by SLUG are directly or indirectly involved in cell–cell adhesion and their inhibition thus may induce dislodging of the cells and metastasis [3].

The exact mode of action of human SLUG is not known. While the C-terminal zinc-finger domains of SLUG are responsible for DNA binding, the N-terminal domain are predicted to be responsible for the recruitment of co-repressor molecules at the target gene promoter. SLUG has a domain with 20 amino acid residues at its N-terminus known as SNAG domain. The SNAG domain was originally characterized in the growth factor independence-1 (Gfi-1) oncoprotein, where it forms part of the first 20 amino acids that suffice for transcriptional repression [2,4]. It is proposed that SLUG binds to the E2-box sequence of the DNA through its C-terminal zinc-finger domains and then

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recruits either CtBP1 [4,8] or a SNAG-domain binding protein (e.g., Sin3A) as a co-repressor. The co-repressor then recruits histone deacetylase (e.g., HDAC1) at the promoter to silent the target gene expression by chromatin remodeling [8]. Here we show evidence that hSLUG does not interact directly with hCtBP1.

Materials and methods

cDNA amplifications and cloning. The ORF of hSLUG was amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA) from its cDNA (8) using 5′CAACATATGATGCCGCGCTCC-3' and 5′-CAAGTCGACG TGTGCTACACAGCAGCC-3' which contained *Nde*I and *Sal*I sites at the 5′-ends, respectively. The PCR product (831 bp) was purified and cloned into pCR-4-TOPO (Invitrogen, Carlsbad, CA). Purified pCR-4-TOPO-hSLUG plasmid DNA was digested with *Nde*I and *Sal*I and cloned into the *Nde*I/*Sal*I sites of the bait vector pGBKT7 (BD Biosciences Clontech, Palo Alto, CA). This gave us pGBK-hSLUG clone. Human CtBP1 (hCtBP1) cDNA was a gift from Prof. G. Chinnadurai of Saint Louis University Medical Center, St. Louis, MO. The ORF of hCtBP1 was amplified using following primers: 5′CAACATATGATGGGCA GCTCG-3′ and 5′CAAATCGATCAACTGGTCACT-3′. The PCR product (1.3 kb) was purified and cloned into pCR-4-TOPO (Invitrogen) and cut out of this plasmid with *Nde*I and *Eco*R I. Next, the CtBP1 fragment was cloned into the *Nde*I and *Eco*RI sites in the multiple cloning site of the prey vector pGADT7 (BD Biosciences Clontech). This gave us pGAD-hCtBP1. The inserts of the pGAD-hCtBP1 and pGBK-hSLUG constructs were sequenced to confirm that the ORFs are in frame with their respective fusion partners.

Yeast 2-hybrid analysis.Saccharomyces cerevisiae strain AH109 (BD Biosciences Clontech) was co-transformed with pair wise combinations of bait and prey vectors with lithium acetate (Matchmaker System 3; BD Biosciences Clontech), as described in the manufacturer's protocol. The yeast strain AH109 was co-transformed with Gal4 DBD-hSLUG fusion construct in pGBKT7 together with a Gal4 activation domain-hCtBP1 fusion construct in pGADT7. Interactions between the proteins, which tethered both domains of GAL4 together, were identified by growth of plasmid-carrying cells on minimal medium without leucine, tryptophan, histidine and adenine and by α-galactosidase activity.

Co-immunoprecipitation experiments. Epitope-tagged proteins (myc-hSLUG and HA-hCtBP1) were expressed from the bait (hSLUG-pGBKT7) and the prey (hCtBP1-pGADT7) vectors using the reagents from the rabbit reticulocyte extract-based TNT T7 Quick-coupled Transcription/ Translation System; (Promega, Madison, WI). Equal volumes of radiolabeled (^{35}S-methionine) proteins, myc-hSLUG and HA-hCtBP1, were mixed together and incubated at 25 °C for 1 h and were subjected to immunoprecipitation with either rabbit polyclonal HA antibodies or mouse c-Myc monoclonal antibodies using the MATCHMAKER Co-IP kit (BD Biosciences Clontech) according to the manufactures protocols. Immunoprecipitated proteins were resolved on NuPAGE 4–12% Bis-Tris gradient gels (Invitrogen) and analyzed by autoradiography. In some experiments, we preincubated the hSLUG with 20 fmols of human BRCA2 gene silencer DNA (8) or a pRL-Null (Promega) plasmid construct containing a 641-bp human claudin 7 gene promoter that has seven E2-box sequences before the binding reaction. We also performed the binding reaction at 37 °C for 1 h.

Results and discussion

Human SLUG has the potential consensus CtBP1 binding site

CtBP1 belongs to the group of proteins that comprise a distinct class of corepressors that interact with a subset of transcription factors through a short sequence motif, PXDLSX[R/K], where X is any amino acid [4,12]. Human SLUG protein has a sequence element [PSDTSSK] similar to this CtBP1 binding consensus sequences at 91–97 positions (Fig. 1). While characterizing the mode of hSLUG in mediating cell cycle stage specific silencing of human BRCA2 gene expression we investigated whether hCtBP1 is associated with this repression [8]. Chromatin immunoprecipitation analysis suggested that hSLUG is co-localized at the 221 bp BRCA2 gene silencer with hCtBP1 [8]. We postulated that hSLUG directly or with the help of an adapter protein binds to hCtBP1 at the promoter of SLUG-regulated genes. Apart from the putative CtBP1 binding motif hSLUG also have a N-terminal SNAG domain (Fig. 1), which may bind to co-repressor molecules like Sin3A [2,3]. We have not yet explored whether hSLUG directly binds to Sin3A.

Yeast 2-hybrid analysis suggests that hSLUG does not bind directly to hCtBP1

To verify whether hSLUG indeed binds directly with hCtBP1 we utilized the yeast two-hybrid techniques (Fig. 2). hSLUG bait constructs were co-transformed into yeast with a prey construct encoding hCtBP1 fused to the Gal4 activation domain. As a control, the bait constructs were co-transformed with the empty vector to confirm that the hSLUG bait constructs were unable to activate reporter genes in the absence of an interaction. A positive interaction was assessed by the ability of the yeast to grow on SD medium lacking tryptophan, leucine, adenine, and histidine (Fig. 2). Unexpectedly, hSLUG did not interact with hCtBP1 whereas the positive control proteins (SV40 T4 antigen and a P53 protein fragment) did show binding (Fig. 2). Verification of the nucleotide sequences of the bait and the prey constructs, reversion of the bait and prey, and the evaluation of the expressions of the hybrid mRNAs and

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MPRSFLVKKHFNASKKPNYSELDTHTVIISPYLYESYSMPVIPQPEILSSGAYSPITVWT  60
TAAPFHAQLPNGLSPLSGYSSSLGRVSPPPPSDTSSKDHSGSESPISDEEERLQSKLSD 120
PHAIEAEKFQCNLCNKTYSTFSGLAKHKQLHCDAQSRKSFSCKYCDKEYVSLGALKMHIR 180
THTLPCVCKICGKAFSRPWLLQGHIRTHTGEKPFSCPHCNRAFADRSNLRAHLQTHSDVK 240
KYQCKNCSKTFSRMSLLHKHEESGCCVAH 269
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Fig. 1. Amino acid sequence of human SLUG protein showing the putative domains. The N-terminal 30 amino acid residues constitute the SNAG domain (shown in green). The putative CtBP1 binding sequence is shown in blue. Underscored amino acid residues indicate the zinc-finger domains. The cysteine and histidine residues of the zinc-fingers are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

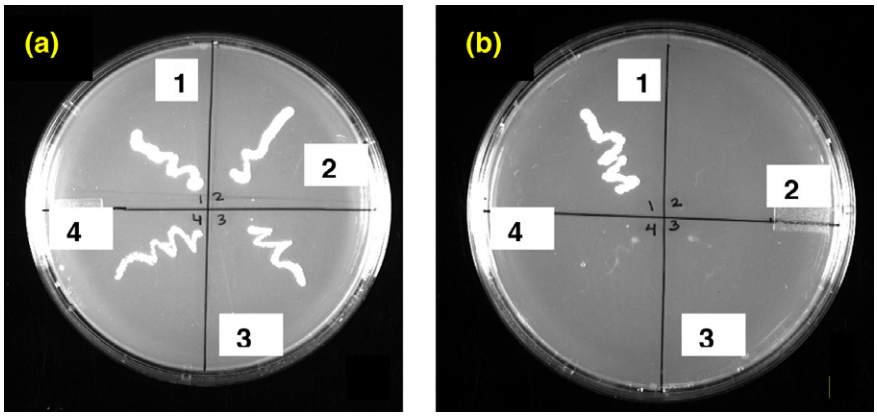

Fig. 2. Yeast-2-hybrid analysis data. Growth of yeast cells on minimal agar plate containing selective dropout medium is shown. The left agar plate (a) is with selective medium (SD medium without leucine and tryptophan) for the growth of the transformants (no insert in the vectors are necessary for the growth). The plate in the right (b) the selective medium (SD medium without leucine, tryptophan, histidine, and adenine) only allowed growth of the yeast cells if the cloned bait protein and the prey protein physically interact. The bait and prey protein pairs in different segments in the photograph are as follows: (1) pGADT7-SV40 T-antigen/pGBKT7-P53 (positive control); (2) blank vectors (negative control); (3 and 4) pGBKT7-hSLUG/pGADT7-hCtBP1.

proteins in the transfected yeast cells were performed to ensure the experimental procedures.

In vitro co-IP analysis confirms that hSLUG does not physically interact with hCtBP1

To further evaluate whether Myc-tagged hSLUG can directly bind with HA-tagged hCtBP1, we synthesized these proteins as ^{35}S-methionine labeled in an *in vitro* transcription and translation system (see Materials and methods). The proteins were mixed together and incubated for 1 h at 25 or 37 °C. The proteins were then precipitated with HA or Myc antibody. The autoradiogram showed no apparent direct binding of hSLUG with hCtBP1 (Fig. 3). Under the conditions of the experiment two proteins known to bind to each other, SV40 T4 antigen and a P53 protein fragment, showed binding (Fig. 3). hSLUG did not show any binding to hCtBP1 either at 25 °C or at 37 °C.

These data suggests that despite the presence of the consensus hCtBP1 binding sequence in the hSLUG protein, they fail to bind each other. The possibilities for the mediation of hSLUG action thus may be through a adapter protein that bridges between hSLUG and hCtBP1. Direct recruitment of other co-repressor protein by the SNAG domain of hSLUG (Fig. 1) is another likely possibility. Our data are consistent with the observation that the N-terminal 20–30 amino acid residues of hSLUG is sufficient to mediate its repressor function [4]. Further detail analysis of the protein–protein interfaces in the hSLUG repressor complex will help us to understand the mode of action of this critical regulator of cell growth and metastasis.

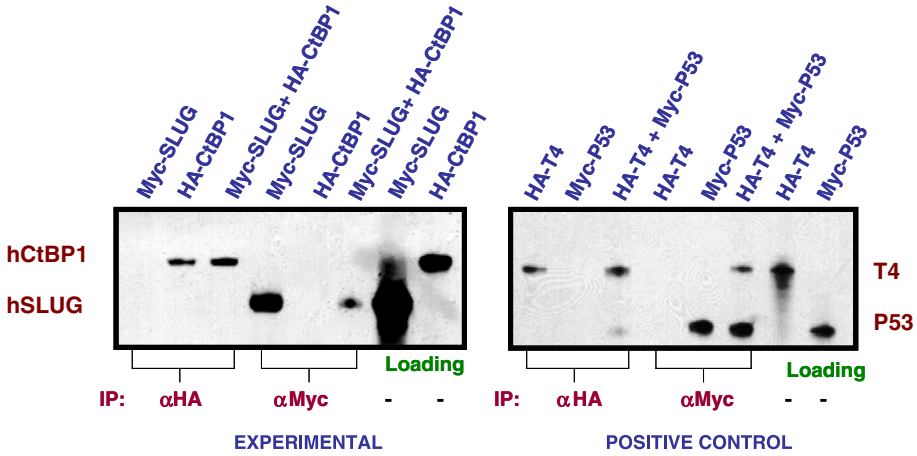

Fig. 3. Co-immunoprecipitation data. Autoradiograms containing the ^{35}S-labeled proteins immunoprecipitaed from the binding reactions are shown. Labeling at the top of the autoradiograms indicates the ^{35}S-labeled proteins in the binding reaction. The Myc-hSLUG is ~31 kDa and HA-hCtBP1 is ~50 kDa. The positive control panel shows interactions between the HA-tagged SV40 T4 antigen (65 kDa) and the Myc-tagged P53 fragment (35 kDa) as was analyzed similarly as the experimental proteins. Loading panels shows 1/10 of the respective proteins used in each binding assay.

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References

[1] Y. Uchikado, S. Natsugoe, H. Okumura, T. Setoyama, M. Matsumoto, S. Ishigami, T. Aikou, Slug Expression in the E-cadherin preserved tumors is related to prognosis in patients with esophageal squamous cell carcinoma, Clin. Cancer Res. 11 (2005) 1174–1180.

[2] A. Barrallo-Gimeno, M.A. Nieto, The Snail genes as inducers of cell movement and survival: implications in development and cancer, Development 132 (2005) 3151–3161.

[3] M.A. Nieto, The snail superfamily of zinc-finger transcription factors, Nat. Rev. Mol. Cell. Biol. 3 (2002) 155–166.

[4] K. Hemavathy, S.C. Guru, J. Harris, J.D. Chen, Y.T. Ip, Human Slug is a repressor that localizes to sites of active transcription, Mol. Cell. Biol. 20 (2000) 5087–5095.

[5] K.M. Hajra, D.Y. Chen, E.R. Fearon, The SLUG zinc-finger protein represses E-cadherin in breast cancer, Cancer Res. 62 (2002) 1613–1618.

[6] S. Chen, J. Ye, I. Kijima, Y. Kinoshita, D. Zhou, Positive and negative transcriptional regulation of aromatase expression in human breast cancer tissue, J. Steroid Biochem. Mol. Biol. 95 (2005) 17–23.

[7] W.S. Wu, S. Heinrichs, D. Xu, S.P. Garrison, G.P. Zambetti, J.M. Adams, A.T. Look, Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma, Cell 123 (2005) 641–665.

[8] M.K. Tripathi, S. Misra, S.V. Khedkar, N. Hamilton, C. Irvin-Wilson, C. Sharan, L. Sealy, G. Chaudhuri, Regulation of BRCA2 gene expression by the SLUG repressor protein in human breast cells, J. Biol. Chem. 280 (2005) 17163–17171.

[9] O.M. Martinez-Estrada, A. Culleres, F.X. Soriano, H. Peinado, V. Bolos, F.O. Martinez, M. Reina, A. Cano, M. Fabre, S. Vilaro, The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells, Biochem. J. 394 (2006) 449–457.

[10] F.E. Turner, S. Broad, F.L. Khanim, A. Jeanes, S. Talma, S. Hughes, C. Tselepis, N.A. Hotchin, Slug regulates integrin expression and cell proliferation in human epidermal keratinocytes, J. Biol. Chem. 281 (2006) 21321–21331.

[11] M.K. Tripathi, S. Misra, G. Chaudhuri, Negative regulation of the expressions of cytokeratins 8 and 19 by SLUG repressor protein in human breast cells, Biochem. Biophys. Res. Commun. 329 (2005) 508–515.

[12] G. Chinnadurai, CtBP, an unconventional transcriptional corepressor in development and oncogenesis, Mol. Cell. 9 (2002) 213–224.

SLUG-dependent modulation of Claudin 7 gene expression in metastatic human breast tumor cells

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SLUG is a zinc finger transcriptional repressor protein that mediates its action through the binding to E2-box sequences (5′-CACCTG-3′) at the promoters of its target genes. SLUG and the other member of its family, SNAIL, are known to down regulate the expressions of many cell adhesion molecules including E-cadherin, occludin, claudins, desmoplakins and desmogleins. While SNAIL is reported to play a major role in these regulations in the non-breast cells, SLUG seems to be the major player in the human breast cells. We recently have reported that SLUG also regulates the expression of the tumor suppressor protein BRCA2 and cytokeratins 8 and 19 in human breast cells. An interesting aspect to add is that all the highly invasive human breast tumor cell lines we have studied express high levels of SLUG whereas the non-invasive breast cells are either SLUG negative or express very little of this protein. Our notion is that a high level of SLUG protein in the breast epithelial cells inhibits the expression of BRCA2 thus promoting unfettered growth of the cells as well as the inhibition of the cell adhesion molecules by SLUG helps the proliferating tumor cells to transform to the mesenchymal cells and ultimately to metastasize. We report here that unlike other epithelial cells, which regulates claudin 7 gene expression by SNAIL, in human breast epithelial cells claudin 7 is regulated by SLUG. SLUG works through the E-box sequences located at the claudin 7 gene promoter. Claudin 7 gene expression depends upon the SLUG status of the cells and is related to the invasive potential of the cells. We are also testing Penetratin 1-tagged molecular decoy against claudin 7 promoter to block the interaction of SLUG with the E2-box to evaluate whether these decoys can influence the function of SLUG inside the invasive breast tumor cells. The study adds to the central role of the transcriptional repressor protein SLUG in the modulation of the proliferation and metastasis of human breast tumor cells. Supported by the DOD-CDMRP IDEA Grant #W81XWH-06-1-0466 and the NCI SPORE subproject grant # 3 P50 CA098131 03S1 to GC.

Differential contribution of distinct E2-box elements in the dual regulation of human claudin 7 gene promoter by SNAIL and SLUG in human breast cells

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SLUG and SNAIL are the members of a family of zinc finger transcriptional repressors, which mediates their actions through the binding to E2-box sequences (5′-CACCTG-3′/3’-GTGGAC-5’) at the promoters of their target genes. Since both SNAIL and SLUG are implicated in the mediation of the epithelial to mesenchymal transition (EMT) by regulating the expressions of many cell-cell adhesion proteins, we studied how these repressors coordinate their regulation of such a gene in the human breast cells. We studied the promoter of the human claudin 7 gene because it has seven potential SLUG/SNAIL regulatable E2-box sequences. We used MDA-MB-231 (SLUG+/SNAIL+) and MDA-MB-468 (SLUG-/SNAIL+) human breast cancer cells for this study. We amplified from human genomic DNA the 641 bp (-345 to +296) claudin 7 gene promoter sequences and cloned it into pRL-NULL vector for promoter activity assay. Deletion and site-directed mutagenesis were performed to evaluate the relative role of individual E2-box sequences towards the expression of the *Renilla* luciferase gene. We found that claudin 7 is not expressed in SLUG-positive cells. Over expression of SLUG in the SLUG-negative MDA-MB-468 cells also abrogated claudin 7 gene expression. The activity of the claudin 7 gene promoter is 30-35 fold higher in the SLUG-negative cells as compared to the SLUG-positive cells. Deletion of two of the seven E2 boxes stimulated the activity of the promoter 2-4 folds both in the SLUG-positive and SLUG-negative cells. We postulate that along with SLUG, SNAIL is also regulating human claudin 7 gene expression in the breast cells. We are developing an inducible knock down system for SNAIL in the human breast cells to further evaluate this notion. The study will add to the central role of the transcriptional repressor proteins SNAIL and SLUG in the modulation of the proliferation and metastasis of human breast tumor cells. Supported by the DOD-CDMRP IDEA Grant #W81XWH-06-1-0466 and the NCI SPORE subproject grant # 3 P50 CA098131 03S1 to GC.